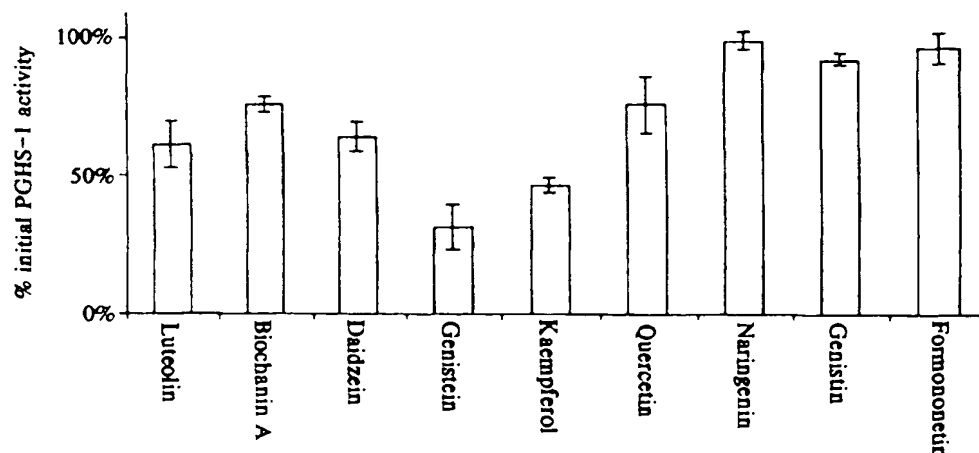




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(54) Title: METHOD FOR INHIBITING CYCLOOXYGENASE AND INFLAMMATION USING CHERRY BIOFLAVONOIDS



## (57) Abstract

A method for inhibiting cyclooxygenase enzymes and inflammation in a mammal using a cherry or cherry anthocyanins, bioflavonoids, and phenolics is described. In particular, a mixture including the anthocyanins, the bioflavonoids, and the phenolics is described for this use.

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METHOD FOR INHIBITING CYCLOOXYGENASE AND INFLAMMATION  
USING CHERRY BIOFLAVONOIDS

5 The present invention relates to a method of use of at least one compound  
obtained from cherries as cyclooxygenase (COX-1 and COX-2) inhibitors. In  
particular, the present invention provides a natural cherry composition containing a  
mixture of anthocyanins, bioflavonoids and phenolics for use as anti-inflammatory  
agents as a result of inhibition of the cyclooxygenase enzymes.

10 Many plant-derived compounds may impart important positive pharmacological  
or "nutraceutical/phytoceutical" traits to foods by way of their abilities to serve as  
antioxidants by maintaining low levels of reactive oxygen intermediates, as anti-  
inflammatory agents by inhibiting prostaglandin synthesis, or as inhibitors of enzymes  
involved in cell proliferation. These activities may be important in ameliorating chronic  
15 diseases including cancer, arthritis, and cardiovascular disease (Kinsella et al., Food  
Tech. 85-89 (1993)). Thus, with natural products, the dietary supplement/food  
industry and nutraceutical/phytoceutical companies have the opportunity to employ  
compounds that can not only enhance food stability as effectively as synthetic  
antioxidants, but can also offer significant health benefits to the consumer.

Cherries are thought to have beneficial health properties in general.  
20 Consumption of cherries was reported to alleviate arthritic pain and gout (Hamel, P.B.,  
et al. Cherokee Plants 28: Herald: Raleigh, N.C. (1975)) although there is no evidence  
for its active components or mode of action. These beneficial effects may be partially  
associated with the abundance of anthocyanins, the glycosides of cyanidin.

*Prunus Cerasus L.* (Rosaceae), cv. MONTMORENCY is the major tart cherry  
25 commercially grown in the United States. In order to challenge the MONTMORENCY  
monoculture, a new cultivar, BALATON® tart cherry (*Ujferbertoi furtos*), was  
introduced into the United States in 1984, and has been tested in Michigan, Utah, and  
Wisconsin. BALATON produces fruits darker than MONTMORENCY does.

Colorants like anthocyanins have been regarded as the index of quality in tart  
30 cherries. Most importantly, recent results showed that anthocyanins such as  
cyanidin-3-glucoside have strong antioxidant activities (Tsuda, T., et al, J. Agric. Food  
Chem. 42:2407-2410 (1994)).

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Early studies have showed that MONTMORENCY cherry contains the anthocyanins cyanidin-3-gentiobioside and cyanidin-3-rutinoside (Li, K. C., et al., J. Am. Chem. Soc. 78:979-980 (1956)). Cyanidin-3-glucosylrutinoside was also found in six out of the seven sour cherry varieties (Harborne, J. B., et al., Phytochemistry 3:453-463 (1964)). Dekazos (Dekazos, E.D., J. Food Sci. 35:237-241 (1970)) reported anthocyanin pigments in MONTMORENCY cherry as peonidin-3-rutinoside, peonidin and cyanidin along with cyanidin-3-sophoroside, cyanidin-3-rutinoside and cyanidin-3-glucoside. However, cyanidin-3-glucosylrutinoside as well as cyanidin-3-glucoside, cyanidin-3-sophoroside and cyanidin-3-rutinoside were identified as main pigments in sour cherries. Using HPLC retention values, Chandra et al. (Chandra, A., et al., J. Agric. Food Chem. 40:967-969 (1992)) reported that cyanidin-3-sophoroside and cyanidin-3-glucoside were the major and minor anthocyanins, respectively, in Michigan grown MONTMORENCY cherry. Similarly, cyanidin-3-xylosylrutinoside was detected as a minor pigment in MONTMORENCY cherry (Shrikhande, A. J. and F. J. Francis, J. Food Sci. 38:649-651 (1973)).

In the prior art, production of pure anthocyanins (compounds 1-3 of Figure 1) from BALATON and MONTMORENCY cherry juices was carried out first by adsorbing the pigment on an AMBERLITE XAD-2 (Sigma Chemicals) column (Chandra, A., et al., J. Agric. Food Chem. 41:1062-1065 (1993)). The column was washed with water until the eluant gave a pH of approximately 7.0. The adsorbed pigments along with other phenolics were eluted with MeOH. The resulting crude anthocyanins were fractionated and purified by C-18 MPLC and HPLC, respectively, to afford pure anthocyanins for spectral studies. Purification of 500 mg crude MONTMORENCY anthocyanins from AMBERLITE XAD-2 yielded 60 mg of pure anthocyanins 1-3 compared to 391.43 mg from BALATON. This research indicated that crude anthocyanins from MONTMORENCY obtained from the XAD-2 contained a high percentage of other organic compounds. There was no attempt to use the crude mixture of phenolics and anthocyanins for any purpose. U.S. Patent Nos, 5,266,685 to Garbutt, 5,665,783 to Katzakian et al. and 5,817,354 to Mozaffar describe various adsorbent resins and their use for isolating unrelated products. These patents are only illustrative of the general state of the art in the use of adsorbent resins.

Cyclooxygenase (COX) or prostaglandin endoperoxide-H synthase (PGHS-1, PGHS-2 or COX-1/COX-2) enzymes are widely used to measure the anti-

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inflammatory effects of plant products (Bayer, T., et al., *Phytochemistry* 28: 2373-2378 (1989); and Goda, Y., et al., *Chem. Pharm. Bull.* 40: 2452-2457 (1992)). Cox enzyme is the pharmacological target site for the nonsteroidal anti-inflammatory drug discovery (Humes, J., et al., *Proc. Natl. Acad. Sci. U.S.A.* 78: 2053-2056 (1981); and Rome, L. H., et al., *Proc. Natl. Acad. Sci. U.S.A.* 72: 4863-4865 (1975)). Two isozymes of cyclooxygenase involved in prostaglandin synthesis are cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), respectively (Hemler, M., et al., *J. Biol. Chem.* 25: 251, 5575-5579 (1976)). It is hypothesized that selective COX-2 inhibitors are mainly responsible for anti-inflammatory activity (Masferrer, J. L., et al., *Proc. Natl. Acad. Sci. U.S.A.* 91: 3228-3232(1994)). Flavonoids are now being investigated as anti-inflammatory substances as well as their structural features for cyclooxygenase (COX) activity. The 5,7-dihydroxyflavone, galangin with an  $IC_{50}$  of 5.5  $\mu$ M, was found to be the most active cyclooxygenase inhibitory flavonoid (Wurm, G., et al., *Deutsche Apotheker Zeitung* 122: 2062-2068 (1982)). Flavonoids with an ortho-dihydroxy in ring A or B were stronger inhibitors than those with a free 3-OH group Murm, G., et al., *Deutsche Apotheker Zeitung* 122: 2062-2068 (1982); and Baumann, J., et al., *Prostaglandins* 20: 627-640 (1980)). The C<sub>2</sub>-C<sub>3</sub> double bond, which determines the coplanarity of the hetero rings appears to be a major determinant of COX activity (Wurm, G., et al., *Deutsche Apotheker Zeitung* 122: 2062-2068 (1982)). Certain prenylated flavonoids, such as morusin, were also active, because of their higher lipophilicity (Kimura, Y., et al., *Chem. Pharm. Bull.* 34: 1223-1227 (1986)). Also, unsubstituted flavone is a good COX inhibitor (Mower, R. L., et al., *Biochem. Pharmacol.* 33: 357-364 (1984); and Welton, A. F., et al., *Prog. Clin. Biol. Res.* 213: 231-242 (1986)). Most of the flavanones studied in the past did not show significant COX inhibition, except for the flavanone-3-ol, silibinin (Kalkbrenner, F., et al., *Pharmacology* 44: 1-12 (1992)). However, the structure-activity relationships of isoflavonoids are not reported.

There is a need for natural product derived compositions for use as cyclooxygenase inhibitors and as anti-inflammatory agents.

## SUMMARY OF THE INVENTION

The present invention relates to a method for inhibiting cyclooxygenase or prostaglandin H synthase enzymes that comprises providing at least one compound obtainable from a cherry with at least one of the enzymes to inhibit the enzymes.

5 Further, the present invention relates to a method for inhibiting cyclooxygenase or prostaglandin H synthase enzymes that comprises providing at least one bioflavonoid compound obtainable from a cherry with at least one of the enzymes to inhibit the enzymes.

10 Further, the present invention relates to a method for inhibiting inflammation in a mammal that comprises administering at least one compound obtainable from a cherry to the mammal to inhibit inflammation.

Further, the present invention relates to a method for inhibiting inflammation in a mammal that comprises administering at least one bioflavonoid, anthocyanin or phenolic compound obtained from a cherry to the mammal to inhibit the inflammation.

15 The present invention relates to a method for inhibiting inflammation in a mammal that comprises administering cyanidin to the mammal to inhibit inflammation.

Also contemplated is a food supplement having anti-inflammatory properties wherein the food supplement comprises a cherry extract having an anti-inflammatory activity greater than the anti-inflammatory activity found in the natural cherry fruit.

20 Also provided is a method of inhibiting COX-2 activity in a cell comprising contacting the cell with a cherry fruit extract having an anti-inflammatory activity greater than the anti-inflammatory activity found in the natural fruit. Certain aspects of the invention contemplate the cell being a mammalian cell. Other embodiments contemplate that the cell is a human cell. Particular embodiments contact the cell with the cherry fruit extract *in vitro*. Others contemplate contacting the cell *in vivo*. In preferred aspects, these methods use a food or food supplement that is provided in the form of a capsule, a tablet, a syrup, a beverage, a powder or the like.

25 Another aspect of the invention provides a method of treating an inflammatory response in an animal comprising administering to the animal a composition comprising a cherry fruit extract having an anti-inflammatory activity greater than the anti-inflammatory activity found in the natural cherry fruit and a pharmaceutically acceptable diluent or excipient.

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In specific embodiments, the inflammatory response may be selected from the group consisting of arthritis, pain, an allergic rash, inflammatory bowel syndrome, and asthma. Of course, these are exemplary inflammatory diseases and it is contemplated that the present inventions may provide an excellent herbal remedy for any disorder resulting from an inflammatory response. It should be understood that the food or food supplements described herein will be useful when being taken alone or in combination with other anti-inflammatory remedies.

In those embodiments where the food supplements are taken in combination with other remedies, the present method contemplates further administering to the animal an anti-inflammatory agent selected from the group consisting of salicylates, glucocorticoids, para-aminophenol derivatives, opioids, indomethacin, sulindac, fenamates, propionic acid derivatives and oxicams. The present supplements may be provided in any of the solid or liquid dosage forms, including, but not limited to, hard or soft gelatin capsules, other capsules, tablets, solutions, suspensions, syrups, beverages or a powders.

Yet another embodiment of the present invention describes a nutraceutical composition comprising an extract of cherry, wherein the nutraceutical provides relief from pain when ingested or otherwise applied to an organism suffering from pain. Specifically, the pain may be the pain of arthritis, menstrual cramps, headaches, insect bites or an allergic rash.

The term "anthocyanins" includes the color producing compounds contained in cherries. For the purpose of this application this includes the aglycone cyanidin.

The term "bioflavonoids" means the isoflavonoid and flavonoid compounds contained in cherries.

The term "phenolics" refers to compounds with a phenyl group and having one or more hydroxyl groups.

The compounds isolated from cherries are most useful with living material. The living material can be in an animal or human. It can also be in tissue culture.

It is therefore an object of the present invention to provide a cherry compound that can be used as cyclooxygenase inhibitors and anti-inflammatory agents. Further, it is an object of the present invention to provide a method for isolating the cherry compound on a commercial scale. Finally, it is an object of the present invention to provide a natural source compound that is economical to prepare and easy to use.

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Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from the following description and the drawings.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the structure of select anthocyanins (colorants) that have been isolated from BALATON and MONTMORENCY cherries. The aglycon cyanidin has a hydroxyl group at position 3.

Figures 2 and 3 are drawings showing the major bioflavonoids isolated from the cherries.

Figure 4 shows select phenolics isolated from tart cherries.

Figure 5 shows the steps in a method for isolating anthocyanins, bioflavonoids, and phenolics from cherries.

Figure 6 is a schematic drawing showing equipment that may be used in the method shown in Figure 5.

Figure 7 is a dose-response curve for the inhibition of the human PGHS-1 enzyme by cyanidin. The anti-inflammatory activity of cyanidin was estimated by its ability to inhibit the cyclooxygenase activity of the PGHS-1 enzyme. Cyanidin gave an  $IC_{50}$  value of 90  $\mu M$  for PGHS-1 enzyme, while the NSAIDs aspirin, naproxen, and ibuprofen gave  $IC_{50}$  values of 1050, 11, and 25  $\mu M$ , respectively.

Figure 8 is a dose-response curve for the inhibition of PGHS-1 and PGHS-2 enzymes by cyanidin. Cyanidin gave  $IC_{50}$  values of 90 and 60  $\mu M$  for PGHS-1 and PGHS-2 enzymes, respectively.

Figure 9 is a graph showing the inhibitory effect of PGHS-1 (COX-1) by flavonoids and isoflavonoids at 200  $\mu M$  concentrations. Data is expressed as mean  $\pm$  S.E. of triplicate. Kaempferol 3-rutinoside, 3'-methoxy kaempferol 3-rutinoside, 5,8,4'-trihydroxy-6,7-dimethoxyflavone and quercetin were not active at 1000  $\mu M$  concentrations.

Figure 10 is a graph showing dose response curves for the inhibition of the PGHS-1 enzyme (COX-1) by flavonoids from BALATON tart cherries compared to the



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non-steroidal anti-inflammatory drugs, naproxen, aspirin, and ibuprofen. The  $IC_{50}$  of kaempferol, quercetin, luteolin, aspirin, naproxen and ibuprofen are 180, 350, 300, 1050, 11 and 25  $\mu M$ , respectively. Data is expressed as mean  $\pm$  S.E. of triplicate.

Figure 11 are graphs showing dose response curve for the inhibition of the PGHS-1 enzyme (COX-1) by isoflavonoids from BALATON tart cherries compared to the non-steroidal anti-inflammatory drugs, naproxen, aspirin and ibuprofen. The  $IC_{50}$  of daidzein, biochanin A, genistein, aspirin, naproxen and ibuprofen are 400, 350, 80, 1050, 11 and 25  $\mu M$ , respectively. Data is expressed as mean  $\pm$  S.E. of triplicate.

#### DESCRIPTION OF PREFERRED EMBODIMENTS

The isolates obtained from a cherry are preferably prepared as a mixture of anthocyanins, bioflavonoids and phenolics. The isolates may be obtained by a method for producing a mixture comprising anthocyanins, bioflavonoids and phenolics from cherries as a composition that comprises:

- (a) providing an aqueous solution containing the anthocyanins, bioflavonoids and phenolics from the cherries;
- (b) removing the anthocyanins, bioflavonoids and phenolics onto a resin surface from the aqueous solution;
- (c) eluting the resin surface with an eluant to remove the anthocyanins, bioflavonoids and phenolics from the resin surface; and
- (d) separating the eluant from the anthocyanins, bioflavonoids and phenolics.

The cherries used to produce the isolates can be obtained from the genus *Prunus* and can be sweet, sour (*Prunus avium*, *Prunus cerasus*), and mixtures thereof. Tart cherries contain high levels of malic acid in addition to other organic acids which contributes to the sour taste of tart cherries. The method isolates malic acid and other organic acids containing sugars that can be used in foods to provide tartness and flavor. Most preferred are the BALATON and MONTMORENCY cherries. This invention also contemplates the use of anthocyanins, bioflavonoids and phenolics from new varieties of cherries, especially those that are cultivated to maximize their anthocyanin content.

The isolated mixture of anthocyanins, bioflavonoids and phenolics can be incorporated into any solid or liquid dosage form and used as a natural nutraceutical,

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phytoceutical or dietary supplement. In general, the selected form may provide a total daily dose of the anthocyanins, bioflavonoids, and phenolics of about 1 to 1000 mg. In one embodiment, a daily dose between 1 to 200 mg is provided. In other embodiments, the daily dose may be between 1 to 100 mg, 1 to 50 mg, 5 to 50 mg, 5 to 25 mg, and 10 to 25 mg. In a preferred embodiment, at least 1 mg is provided as the daily dose, and more preferably, at least 5 mg is provided as the total daily dose. As a comparison, one hundred (100) cherries provide about 10 to 100 mg of anthocyanins and bioflavonoids, depending on the type of cherry consumed. Similarly, one hundred cherries provide 1-50 mg of phenolics.

The total daily dose may be comprised of a mixture of anthocyanins, bioflavonoids, and phenolics. Alternatively, the total daily dose may be provided by any one of these three types of compounds or mixtures of any or all of these compounds. For example, in other embodiments, it may be desirable to provide between 0.01 to 200 mg of the anthocyanins, and more preferably, between about 0.01 to 100 mg, 0.01 to 50 mg, 1 to 50 mg, 5 to 50 mg, and 5 to 25 mg of one of more anthocyanins that have been obtained from cherries. Similarly, the bioflavonoids and phenolics individually may be used in same dosage ranges as provided for the anthocyanins directly above. The amount of the anthocyanins, bioflavonoids and phenolics can be adjusted by isolating the individual compounds and blending them together. In one embodiment, a natural mixture of the anthocyanins, bioflavonoids and phenolics is used.

The mixture of anthocyanins, bioflavonoids, and phenolics may be obtained from an extraction method that uses adsorbent resin. The resin has a surface to which the anthocyanins, bioflavonoids and the phenolics are adsorbed. A preferred class of adsorptive resins are polymeric crosslinked resins composed of styrene and divinylbenzene such as, for example, the AMBERLITE series of resins, e.g., AMBERLITE XAD-4 and AMBERLITE XAD-16, which are available commercially from Rohm & Haas Co., Philadelphia, PA. Other polymeric crosslinked styrene and divinylbenzene adsorptive resins suitable for use according to the invention are XFS-4257, XFS-4022, XUS-40323 and XUS-40322 manufactured by The Dow Chemical Company, Midland, Michigan, and the like.

It is preferred to use commercially available, governmentally-approved (where required), styrene-divinyl-benzene (SDVB) crosslinked copolymer resin, (e.g.,

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AMBERLITE XAD-16). Thus, in the preferred embodiment, AMBERLITE XAD-16, commercially available from Rohm and Haas Company, and described in U.S. Patent No. 4,297,220, is used as the resin. This resin is a non-ionic hydrophobic, cross-linked polystyrene divinyl benzene adsorbent resin. AMBERLITE XAD-16 has a  
5    macroreticular structure, with both a continuous polymer phase and a continuous pore phase. In a particularly preferred embodiment, the resin used in the present invention has a particle size ranging from 100-200 microns.

It is contemplated that other adsorbents such as those in the AMBERLITE XAD adsorbent series, which contain hydrophobic macroreticular resin beads, with particle  
10    sizes in the range of 100-200 microns, will also be effective in the methods of the present invention. Moreover, different variations of the AMBERLITES, such as the AMERCHROM CG series of adsorbents, used with particle sizes in the range of 100-200 microns, may also be suitable for use in the present invention. The AMBERLITE XAD-16 is preferred since it can be re-used many times (over 100  
15    times). However, it is contemplated that for food, the use of governmentally-approved resins in the present invention will be considered important and/or desirable.

Any solvent can be used to remove the adsorbed anthocyanins, bioflavonoids and phenolics. Preferred are lower alkanols containing 1 to 4 carbon atoms and most preferred is ethanol (ethyl alcohol) since it is approved for food use. Typically the  
20    ethanol is azeotroped with water; however, absolute ethanol can be used. Water containing malic acid and sugars in the cherries pass through the column. These are collected and can be used in foods as flavors.

The anthocyanins, bioflavonoids and phenolics are preferably obtained from the BALATON and the MONTMORENCY cherries. The composition of the cherries is  
25    in part shown in part by U.S. application Serial No. 08/799,788 filed February 12, 1997 and in part U.S. application Serial No. 60/111,945, filed December 11, 1998 and 60/120,178, filed February 16, 1999. As described in these applications, the Montmorency (*Prunus cerasus*) variety constitutes more than 95% of tart cherry cultivations in Michigan and USA. However, Balaton tart cherry (*P. cerasus*), a new  
30    tart cherry cultivar, is being planted to replace Montmorency in several Michigan orchards. This cherry has higher anthocyanin contents and is regarded as a better variety. Anthocyanin contents of Montmorency and Balaton tart cherries have been reported (Wang, et al., 1997; Chandra et al., 1993). However, a detailed investigation

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of other phenolic compounds in Balaton tart cherry was not carried out before. Early studies have shown that MONTMORENCY cherry contains cyanidin-3-gentiobioside and cyanidin-3-rutinoside (Li, K.C., et al., J. Am. Chem. Soc. 78:979-980 (1956)). Cyanidin-3-glucosylrutinoside was also found in six out of the seven sour cherry varieties (Harbone, J.B., et al., Phytochemistry 3:453-463 (1964)). Dekazos (Dekazos, E.D., J. Food Sci. 35:237-241 (1970)) reported anthocyanin pigments in MONTMORENCY cherry as peonidin-3-rutinoside, peonidin and cyanidin along with cyanidin-3-sophoroside, cyanidin-s-rutinoside and cyanidin-3-glucoside. However, cyanidin-3-glucosylrutinoside as well as cyanidin-3-gluocoside, cyanidin-3-sophoroside and cyanidin-3-rutinoside were identified as main pigments in sour cherries. Using HPLC retention values, Chandra et al (Chandra, A., et al., J. Agric. Food Chem 40:967-969 (1992)) reported that cyanidin-3-sophoroside and cyanidin-3-glucoside were the major and minor anthocyanins, respectively, in Michigan grown MONTMORENCY cherry. Similarly, cyanidin-3-xylosylrutinoside was detected as a minor pigment in MONTMORENCY cherry (Shrikhande, A.J. and F.J. Francis, J. Food Sci. 38:649-651 (1973)).

The term "carrier" or "bulking agent" is used to mean a composition, which is added to increase the volume of the composition of the purified composition from the cherry. In one embodiment, dried cherry pulp is used as the carrier. In other embodiments, edible starch containing material or protein, such as non-fat dry milk are used as the carrier. Within this group are flour, sugar, soybean meal, maltodextrin and various condiments, such as salt, pepper, spices and herbs, for instance. The bulking agent is preferably used in an amount between about  $10^{-6}$  and  $10^6$  parts by weight of the mixture. The ratio of anthocyanins, bioflavonoids and phenolics to the carrier may be between 0.1 to 100 and 100 to 0.1.

The present invention in certain aspects describes the beneficial intake of a food or food supplement having anti-inflammatory properties wherein the food or food supplement comprises a cherry extract having an anti-inflammatory activity greater than the anti-inflammatory activity found in the natural fruit. Those of skill in the art should understand that such food or food supplement may advantageously be combined with other anti-inflammatory agents. Such additional anti-inflammatory agents will, of course, be secondary to the cherry extracts of the present invention and may be any commonly recognized anti-inflammatory agent. If other anti-inflammatory

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agents are used, the ratio of the cherry extracts to the anti-inflammatory agent(s) may be between 1:0 to 0:1.

The cherry extract and the additional anti-inflammatory agent may be contacted with or exposed to a cell either *in vivo* or *in vitro* to inhibit the COX-activity of the cell.

5 The terms "contacted" and "exposed," when applied to a cell, are used herein to describe the process by which a cherry extract and a second anti-inflammatory agent are delivered to a target cell or are placed in direct juxtaposition with the target cell. To achieve a beneficial effect, both agents may be delivered to a cell in a combined amount effective to inhibit COX activity, decrease inflammation, decrease the  
10 production of the inflammation causing prostaglandins or other such effect that will decrease the inflammatory response in a cell or an individual subject in which the cell is located.

Anti-inflammatory agents are well known to those of skill in the art and include agents such as salicylic acid derivatives (e.g. aspirin) paraminophenol derivative (e.g.  
15 acetaminophen) indole and indene acetic acids (indomethacin, sulindac and etodalac) heteroaryl acetic acids (tolmetin diclofenac and ketorolac, aryl propionic acid derivatives (ibuprofen, naproxen, ketoprofen, keopren, fenopren, oxaprozine), anthranilic acids (mefenamic acid, meclofenamic acid) enolic acids (piroxicam, tenoxicam, phenylbutazone and oxyphenthazone). These and other anti-  
20 inflammatory agents are well known to those of skill in the art and additional description of these agents may be found in Goodman & Gilman's The Pharmacological Basis of Therapeutics, Ninth Ed. (1996).

The present invention provides a natural food or food supplement made from cherry extracts wherein the food or food supplement comprises an anti-inflammatory  
25 activity that is greater than the anti-inflammatory activity found in the natural cherry. The present invention provides a cherry extract that can be presented in a powdered, liquid, or solid form. Nonlimiting examples include desirable and readily produced forms.

The composition may be introduced into a food in an amount between about  
30 0.1 and 10 mg/gm of the active ingredients of the food. The amount is preferably selected so as to not affect the taste of the food and to produce the most beneficial result. The food can be high (wet) or low moisture (dry) as is well known to those skilled in the art.

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The cherry extract may be in a reconstitutable powder composition that, when reconstituted with, for example, water, milk or some other similar liquid will provide a drink that may be used to provide an anti-inflammatory activity to a subject in need thereof. The powdered composition and drink prepared therefrom are especially  
5 useful as an enterally administered component in a program of pain or inflammation management that utilizes a number of carefully designed products in various forms, *i.e.*, in shake, soup, fruit drink, snack bar and other solid forms such as tablets, gel caps, and the like, which can be mixed and matched over a period of pain management to provide more attractive and, therefore, more effective support to a  
10 patient, particularly those in extended care situations.

In addition to drinks, the cherry extracts of the present invention may be used in foodstuffs. Such cherry extracts may be combined with any other foodstuff, for example, oils containing the extracts of this invention may be used as cooking oil, frying oil, or salad oil and may be used in any oil-based food, such as margarine,  
15 mayonnaise or peanut butter. Grain flour fortified with the compounds of this invention may be used in foodstuffs, such as baked goods, cereals, pastas and soups. Oils containing the cherry extracts and novel anthocyanins, bioflavonoids, and phenolics extracted therefrom can be emulsified and used in a variety of water-based foodstuffs, such as drinks, including drink mixes as discussed above. Advantageously, such  
20 foodstuffs may be included in low fat, low cholesterol or otherwise restricted dietary regimens.

In addition, other flavorings and additives well known to those of skill in the art also, may be added to the formulations to make them more palatable. For example, formulations may contain fruit flavoring, coloring, preservatives and the like.

25 When formulated as a food supplement (also commonly referred to as dietary supplements or nutraceuticals) the composition of the invention may additionally contain a solid carrier such as a gelatin or an adjuvant. The tablet, capsule, and powder may contain from about 0.1 to about 95% by weight of the anti-inflammatory composition of the present invention. In one embodiment, the anti-inflammatory  
30 composition comprises about 0.01 to about 95 % by weight of the total weight of the supplement. More preferably, the anti-inflammatory composition comprises about 0.1 to about 50 %, about 1 to about 25%, or about 1 to about 10% by weight of the total weight of the supplement. When administered in liquid form, a liquid carrier such as

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water, petroleum, oils of animal or plant origin such as peanut oil, mineral oil, soybean oil, or sesame oil, or synthetic oils may be added. Similarly, the liquid form of the nutraceutical compositions contains from about 0.01 to about 95% by weight of the anti-inflammatory compositions of the present invention. More preferably, the nutraceutical compositions contain from about 0.1 to about 50%, about 1 to about 25%, or about 1 to about 10% by weight of the anti-inflammatory composition. The nutraceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additives known to those of skill in the art.

Methods have been developed for extraction and isolation of phytochemicals (Chandra, A., et al., J. Agric. Food Chem. 41:1062 (1992); Wang, H., et al., J. Agric. Food Chem. 45:2556-2560 (1997)) and for rapid screening of antioxidant activity (Arora, A. and G. M., Strasburg, J. Amer. Oil Chem. Soc. 74:1031-1040 (1997)). These methods are being utilized to identify, characterize and test the compounds from BALATON and MONTMORENCY cherries. Juiced cherry tissue was sequentially extracted with hexane, ethyl acetate and methanol. Both methanol and ethyl acetate fractions showed strong antioxidant activity in the screening assay. The ethyl acetate fraction was further purified by silica gel vacuum liquid chromatography to yield four subfractions; the subfraction was further separated into seven fractions by preparative reverse phase HPLC. Figures 2 and 3 show the bioflavonoids isolated from the BALATON cherries. There are thus numerous analogous or homologous compounds in the tart cherries. The anthocyanin components obtained from the juice fraction also have been identified and fully characterized (Chandra, A., et al., J. Agric. Food Chem. 41:1062 (1992); Wang, H., et al., J. Agric. Food Chem. 45:2556-2560 (1997)).

Two novel phenolic compounds were identified:

- I) 1-(3'-4'-dihydroxy cinnamoyl)-2,3-dihydroxy cyclopentane, and
- II) 1-(3'-4'-dihydroxy cinnamoyl)-2,5-dihydroxy cyclopentane.

Other compounds isolated from the ethyl acetate extract of cherry fruits and characterized by spectral methods include: 1-(3'-methoxy, 4'-hydroxy cinnamoyl) quinic acid, 2-hydroxy-3-(2'-hydroxyphenyl) propanoic acid, methyl 2-hydroxy-3-(2'-hydroxyphenyl) propanoate, D(+)-malic acid,  $\beta$ -sitosterol and  $\beta$ -sitosterol glucoside. Figure 4 shows some of the phenolics, which were isolated.

#### Examples 1 and 2

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As shown in Figure 5, individual quick frozen (IQF) cherries (which had been pitted) were defrosted and blended in an industrial WARING blender. The mixture was centrifuged at 10,000 rpm and the juice was decanted. The residue, pulp, was further pressed with cheese cloth to remove any additional juice.

5       The pulp was lyophilized at 15° C. The juice was processed on AMBERLITE XAD-16 HP resin to produce cherry sour, anthocyanins, bioflavonoids and phenolics. The XAD-16 resin, 1 kg, was washed with ethanol (1-2 L) and then washed with water (6 L). The XAD-16 resin was allowed to stand in water for 1 hour before loading into a glass column (10 ID x 90 cm long) with a cotton plug. The packed column was  
10       washed with water (2 L) before loading the juice for separation. 800 mL juice was purified each time. The juice was added onto the surface of the column and allowed to settle with no flow. It was then eluted with water and the first 1 L was discarded. The next 2L of washing was collected, since it contained the cherry juice that was sour since it contained malic acid and sugars from the cherries. The column was then  
15       washed with an additional 4 L of water in the case of BALATON and 5 L for MONTMORENCY cherry juice. Once the cherry juice was collected, the remainder of the washing with water were discarded. The column was then eluted with ethanol (1.3-1.5 L) and collected the red solution containing anthocyanins, bioflavonoids, and phenolics (700-800 ml). The column was then run dry and washed with 10 L of water  
20       before repeating the process many of times (over 100).

The red alcoholic solution was then evaporated under a vacuum (20 milliTorr) to remove ethanol and the aqueous solution, stabilized with 50 ppm ascorbic acid, and lyophilized at 10° C. The red powder was collected and stored.



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Example 1 results:

BALATON cherry

Weight of IQF cherries	15.74 kg
Weight of dried pulp	605 g
Volume of juice	12.16L
Weight of anthocyanins, bioflavonoids and phenolics (red powder)	31.35 g
Volume of sour byproduct (malic acid and sugars)	@ 35 L

Example 2 results:

MONTMORENCY cherry

Weight of IQF cherries	30.45 kg
Weight of dried pulp	895 g
Volume of juice	24.03 L
Weight of anthocyanins, bioflavonoids and phenolics (red powder)	47 g
Volume of cherry by-product (malic acid and sugars)	@ 75 L

The red powders of Examples 1 and 2 were preferably mixed with dried pulp as a carrier and tableted into 1 to 1000 mg tablets including the carrier (1 adult daily dose).

Various food grade acids can be added to the isolated anthocyanins, bioflavonoids and phenolics to prevent decomposition. Preferably, they do not add flavor. Ascorbic acid (vitamin C) is preferred. The acid can be added before or after, preferably before drying of the cherry compounds.

For small scale processing, lyophilization is used to remove water. For larger scale production, drying in an air circulating oven is preferred.

Example 3

As shown in Figure 6, an open vessel 10 is provided with an inlet line 11 and an outlet line 12, with valves 13 and 14, respectively. The resin beads 15 are provided in the open vessel 10. Water is introduced into the vessel 10 and then removed through outlet line 12 and discarded. The cherry juice (without the pulp or pits) as in Example 1 is introduced to the vessel 10 and allowed to stand for 25 minutes. The temperature of the water and juice is between about 20° and 30° C. The cherry juice residue containing malic acid and sugars is then removed through the

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outlet line 12 and retained as a food flavoring. The resin 15 in the vessel is then washed again with water from inlet line 11 and then removed and discarded through outlet line 12. The anthocyanins, bioflavonoids and phenolics on the resin particles are then extracted using 95% ethanol introduced through inlet line 11. The ethanol containing the anthocyanins, bioflavonoids and phenolics is removed from the vessel 10. The ethanol is removed from the anthocyanins, bioflavonoids and phenolics and dried using flash drying under nitrogen. The resulting powder is preferably then mixed with dried cherry pulp or other carrier as in Example 1. The resin particles are washed with water and then the resins and ethanol are recycled many times.

#### Example 4

The anti-inflammatory assays on the anthocyanins and cyanidin were conducted using prostaglandin endoperoxide H synthase-1 and -2 isozymes (PGHS-1, and -2) and were based on their ability to convert arachidonic acid to prostaglandins (PGs). The positive controls used in this experiment were aspirin, naproxen, and ibuprofen. Aspirin gave an  $IC_{50}$  value of 1050  $\mu M$  each against PGHS-1 and PGHS-2 enzymes (Figure 7). Naproxen and ibuprofen gave  $IC_{50}$  values of 11 and 25 nM against PGHS-1 enzyme, respectively (Figure 7). A preliminary experiment with the mixture containing anthocyanins 1-3 (Figure 1) showed PGHS-1 and PGHS-2 activities at 33 ppm concentration. The aglycon cyanidin showed good PGHS-1 and -2 inhibitory activities, with  $IC_{50}$  values of 90 and 60 nM, respectively (Figures 7 and 8). The ratio of  $IC_{50}$  values for PGHS-1 to PGHS-2 was about 0.56 (Figure 8). However, pure anthocyanins 1-3 showed little or no activity against PGHS-1 and PGHS-2 at 300 -nM test concentrations. Higher concentrations of anthocyanins 1 and 2, on the contrary, increased the activity of enzyme. This is probably due to the ability of anthocyanins 1 and 2 to act as oxygen carriers at high concentration and enhance the oxygen uptake. It is noted that anthocyanins are hydrolyzed in the gut of a mammal to cyanidin and other compounds and thus effective *in vivo*.

For measurements of time-dependent inhibition of PGHS-2 enzyme activity by cyanidin, the enzyme was preincubated at 37° C with 15 nM of cyanidin (one-fourth of the concentration of  $IC_{50}$ ) and added to an oxygen electrode chamber with arachidonic acid substrate to initiate the reaction. The results suggest that the rate of inhibition of PGHS-2 did not change with time. The specific inhibition of the PGHS-2 enzyme is a

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major advance in anti-inflammatory therapy because it significantly reduces the adverse effects of nonsteroidal anti-inflammatory drugs (NSAIDs). It is generally believed that ulcerogenic and other adverse properties of NSAIDs result from the inhibition of PGHS-1, whereas the therapeutically desirable effects come from the inhibition of PGHS-2 enzyme.

Similarly, cyanidin showed better anti-inflammatory activity than aspirin in the inflammatory assays. The antioxidant and anti-inflammatory properties of anthocyanins and cyanidin suggest that consumption of cherries may have the potential to reduce cardiovascular or chronic diseases in humans.

In particular, arachidonic acid and a microsomal fraction of ram seminal vesicles containing PGHS-1 enzyme suspended in 100 mM Tris pH 7.8 and 300  $\mu$ M diethyldithiocarbamic acid (DDC) as a preservative were purchased from Oxford Biomedical Research (Oxford, MI). Recombinant human PGHS-2 enzyme was initially obtained from Dr. David Dewitt (Department of Biochemistry, Michigan State University, East Lansing, MI) and then purchased from Oxford Biomedical Research (Oxford, MI). Naproxen, ibuprofen, and hemoglobin were purchased from Sigma Chemical Co. (St. Louis, MO). Anthocyanins 1-3 were purified from Balaton tart cherry by HPLC and were identified by  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectral data.

To prepare cyanidin, the anthocyanin mixture containing 1-3 (Figure 1; 500 mg) was stirred with 3N HCl (20 mL) at 80° C for 10 hours. The reaction mixture was purified on a XAD-4 column as in the preparation of anthocyanins. The MeOH solution of cyanidin was evaporated to dryness to yield a red amorphous powder (190 mg) and stored at -30° C until use.

In the anti-inflammatory assay, cyclooxygenase activities were measured by using PGHS-1 enzyme (ca. 5 mg protein/mL in 0.1 M TrisHCl, pH 7.8), a homogeneous protein purified from ram seminal vesicles. Microsomal preparations from recombinant human prostaglandin synthase-2 (COX-2) were obtained from insect cell lysate. Assays were performed at 37° C by monitoring the initial rate of  $\text{O}_2$  uptake using an  $\text{O}_2$  electrode (Yellow Springs Instrument Inc., Yellow Springs, OH). Each assay mixture contained 3 mL of 0.1 M Tris HCl, pH adjusted to 7 by the addition of 6M HCl, 1 mM phenol, 85  $\mu$ g hemoglobin, and 10  $\mu$ M of arachidonic acid. Reactions were initiated by the addition of 5-25  $\mu$ g of microsomal protein in a volume of 15-50  $\mu$ L. Instantaneous inhibition of enzyme activity was determined by

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measuring the cyclooxygenase activity initiated by adding aliquots of microsomal suspensions of PGHS-1 or PGHS-2 (10  $\mu$ M O<sub>2</sub>/min cyclooxygenase activity/aliquot) to assay mixtures containing 10  $\mu$ M arachidonate and various concentrations of the test substances (10-1100  $\mu$ M). The IC<sub>50</sub> values represent the concentrations of the test compound that gave half-maximal activity under the standard assay conditions.

#### Example 5

This is an anti-inflammatory assay for cyclooxygenase inhibition activity of flavonoids and isoflavonoids. Arachidonic acid and microsomal suspensions of PGHS-1 (COX-1) and COX-2 (PGHS-2) were purchased from Oxford Biomedical Research (Oxford, MI, USA). Genistein, genistin, naringenin, quercetin, 5,8,4'-trihydroxy-6,7-dimethoxyflavone, kaempferol-3-rutinoside and 3'-methoxy kaempferol 3-rutinoside were purified from BALATON tart cherry by HPLC and were identified by <sup>1</sup>H- and <sup>13</sup>C NMR spectral data. Daidzein and formononetin were purchased from Research Plus, Inc. (Bayonne, New Jersey, USA). Biochanin A, kaempferol, quercetin, naproxen, ibuprofen and hemoglobin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Luteolin was purchased from Adams Chemical Co. (Round Lake, IL, USA).

For measuring the COX activity, flavonoids or isoflavonoids were dissolved in DMSO to yield 40 mM stock solution and was further diluted to the desired concentration according to the COX-1/COX-2 inhibitory activity of each compound assayed.

Anti-inflammatory assay: COX activities were measured using microsomal suspensions of PGHS-1 and PGHS-2. Microsomal membranes (5 mg protein/mL in 0.1 M Tris HCl, pH 7.4) were prepared and assayed on the same day. COX-1 and COX-2 assay was performed at 37° C controlled by a circulation bath (Model-1166, VWR Scientific Products, Chicago, IL) by monitoring the rate of O<sub>2</sub> uptake using a 5357 Oxygen electrode (INSTECH Laboratory, Plymouth Meeting, PA) (Meade, E. A., et al., J. Biol. Chem. 268 6610-6614 (1993)).

Each assay mixture contained 600  $\mu$ L of 0.1 M Tris-HCl, pH 8.0, 1 mM phenol, 17  $\mu$ g hemoglobin and 10  $\mu$ M arachidonate and were mixed in a microchamber (INSTECH Laboratory, Plymouth Meeting, PA, USA). For anthocyanins and cyanidin pH 7 is preferred to prevent decomposition in absence of additives. Reactions were initiated by adding 5  $\mu$ g of microsomal protein (5  $\mu$ L). Instantaneous inhibition was

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determined by measuring the cyclooxygenase activity initiated by adding microsomal suspensions of PGHS-1 or PGHS-2 in the assay mixtures containing 10  $\mu$ M arachidonate and various concentrations of test compounds. The  $IC_{50}$  values represent the concentrations of inhibitor that gave half-maximal activity under the standard assay conditions. The kinetics of the enzyme activity was monitored by Biological Oxygen Monitor (YSI model 5300, Yellow Springs Instrument CO., Inc., Yellow Springs, Ohio) and collected in Quicklog Data Acquisition and Control computer software (Strawberry Tree Inc., Sunnyvale, CA, USA).

The COX-1/COX-2 activity of BALATON cherry bioflavonoids was determined by monitoring the  $O_2$  uptake. Reactions were initiated by adding PGHS enzyme preparation. One unit of cyclooxygenase represents oxygenation of 1 nmol of arachidonate/min under the standard assay condition by the COX enzyme. This assay was a modification of the assay reported by DeWitt et al. (Dewitt D. L., et al., J. Biol. Chem. 265: 5192-5198 (1990)). 10  $\mu$ M arachidonate has been used for COX-1 assays, because this substrate concentration was reported to give near-maximal COX activity and also permit the detection of enzyme inhibition by lipophilic inhibitors (Meade, E. A., et al., Biol. Chem. 268: 6610-6614 (1993)). This methodology can also be used for COX-2 assay as well using COX-2 enzyme. Three known COX inhibitors, aspirin, ibuprofen and naproxen, were selected as positive controls. COX-1 inhibitory activities of flavonoids, kaempferol, quercetin, luteolin, quercetin 3-rhamnoside, 5,8,4'-trihydroxy-6,7-dimethoxyflavone were compared. Kaempferol 3-rutinoside, 3'-methoxy kaempferol 3-rutinoside and naringenin (Figure 9), and five isoflavonoids, genistein, genistin, daidzein, formononetin and biochanin A (Figure 8).

COX-1/COX-2 inhibitory activities of each compound at different concentrations was calculated by comparing the tangent of  $O_2$  uptake curves of test compounds with that of blank control. Each assay was repeated 3 times and the  $IC_{50}$  values (50 inhibitory concentrations) were calculated by linear regression analysis. The half-maximal inhibitory concentrations of flavonoids and isoflavonoids are shown in Figure 11. Dose response curves for the inhibition of the COX-1 enzyme by flavonoids and isoflavonoids from BALATON tart cherries compared to the non-steroidal anti-inflammatory drugs, aspirin, naproxen and ibuprofen are shown in Figures 10 and 11, respectively.

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Among the flavonoids tested, kaempferol showed the highest COX-1 inhibition, followed by luteolin, quercetin, naringenin and quercetin 3-rhamnoside (Figure 9). In comparing kaempferol with quercetin, it was found that the presence of a hydroxyl group at C<sub>3</sub>' position decreased the COX-1 inhibitory activity (Figure 9). The COX-1 inhibitory activity of kaempferol and quercetin were reported in other model systems (Kalkbrenner, F., et al., Pharmacology 44: 1-12 (1992) ; Hoult, J.R.S., et al., Agents and Actions 42: 787-792 (1988) ; and Moroney, M. A., et al., J. Pharm. Pharmacol. 40: 787-792 (1988)). The OH group at C<sub>3</sub> position is also important for the activity. However, the glycosylation of the OH group, at C<sub>3</sub> decreased the activity considerably. Comparing the COX-1 inhibitory activity of flavones (luteolin) with their corresponding flavanols (quercetin), it can be concluded that the absence of an OH group at C<sub>3</sub> enhanced the COX-1 activity slightly. It is important to note that quercetin 3-rhamnoside was not active in the assay, but reported to have *in vivo* anti-inflammatory activity (Sanchez De Medina, L. H., et al., J. Pharmacol. Exp. Ther. 278: 771-779 (1996)). This may be due to the *in vivo* metabolism of quercetin 3-rhamnoside to quercetin. The C<sub>2</sub>-C<sub>3</sub> double bond, which determines the coplanarity of the hetero-rings in flavonoids and isoflavonoids, was essential for a higher COX inhibitory activity. If the double bond was saturated, the COX-1 inhibitory effect was dramatically decreased as in the case of naringenin (Figure 9). This result is consistent with previous reports (Wurm, G., et al., Deutsche Apotheker Zeitung 122: 2062-2068 (1982); Kalkbrenner, F., et al., Pharmacology 44: 1-12 (1992)). Also, the multiple substituents such as OH and OMe groups in the A ring of the flavonoids caused little or no COX-1 inhibition as demonstrated by the activity of 5,8,4'-trihydroxy-6,7-dimethoxyflavone.

Among the isoflavonoids (Figures 2 and 3), genistein showed the highest COX-1/COX-2 inhibitory activity. The activity was dramatically decreased in genistin, when the 7-OH group in ring A of genistein was glycosylated. Also, the hydroxyl group at C-4' in isoflavonoids is essential for the COX-1/COX-2 inhibitory activity. When 4' -OH groups in genistein and daidzein were methylated, the activity decreased considerably. The 5-OH group in isoflavonoids is also important for COX-1/COX-2 inhibitory effect. These results indicated that C<sub>4</sub>', C<sub>5</sub> and C<sub>7</sub> hydroxyl groups in isoflavonoids are essential for COX-1 inhibition. Comparison of genistein with that of kaempferol indicates that substitutions on ring B and at C<sub>3</sub> of ring C

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enhances COX-1/COX-2 inhibitory effect. In addition to COX-1/COX-2 inhibition, these isoflavonoids and flavonoids also showed good antioxidant activity. Both COX-1 inhibitory and antioxidant activities of these compounds suggests that tart cherries may possess significant health benefits to humans. These bioflavonoids may be partially responsible for the anecdotal claims associated with tart cherries of alleviating pain related to treatment of arthritis and gout.

Thus, several flavonoids and isoflavonoids isolated from BALATON tart cherry were assayed for prostaglandin H endoperoxide synthase (PGHS-1 or PGHS-2) enzyme activity. Genistein showed the highest COX-1 inhibitory activity among the isoflavonoids studied with an  $IC_{50}$  value of 80  $\mu$ M. Kaempferol gave the highest COX-1 inhibitory activity among the flavonoids tested with an  $IC_{50}$  value of 180  $\mu$ M. The structure-activity relationships of flavonoids and isoflavonoids revealed that hydroxyl groups at C<sub>4'</sub>, C<sub>5</sub> and C<sub>7</sub> in isoflavonoids were essential for appreciable COX-1 inhibitory activity. Also, the C<sub>2</sub>-C<sub>3</sub> double bond in flavonoids is important for COX-1 inhibitory activity. However, hydroxyl group at C<sub>3'</sub> position decreased the COX-1/COX-2 inhibitory activity by flavonoids.

#### Example 6

The composition of Examples 1 and 2 were tested for anti-inflammatory activity using cyclooxygenase 1 and 2 (COX-1 and COX-2) in an assay as described in Wang et al., J. Nat. Products 62:294-296 (1999); Wang et al., J. of Ag. and Food Chemistry, 47: 840-844 (1999) and Wang et al., J. of Nat. Products, 62:86-88 (1999) and Examples 4 and 5. The results were that the compositions exhibited anti-inflammatory activities, specifically strong inhibition of COX-1 and COX-2.

It is intended that the foregoing description be only illustrative of the present invention and that the present invention be limited only by the hereinafter appended claims.

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## CLAIMS:

1. Use of at least one compound obtainable from a cherry to inhibit cyclooxygenase or prostaglandin H synthase enzymes.
2. Use of at least one bioflavonoid compound obtainable from a cherry to inhibit cyclooxygenase or prostaglandin H synthase enzymes.
3. The use according to any one of the preceding claims wherein the use is *in vitro*.
4. The use according to claim 1 or 2 wherein the use is *in vivo*.
5. The use according to any one of the preceding claims wherein the compound is from the genus *Prunus*.
6. The use according to any one of claims 1, 2, 3 or 4 wherein the compound is from *Prunus avium*, *Prunus cerasus*, and mixtures thereof.
7. Use of at least one compound obtainable from a cherry to inhibit inflammation in a mammal.
8. The use according to claim 7 wherein the mammal is human.
9. The use according to claim 7 or 8 wherein the compound includes at least one bioflavonoid.
10. The use according to claim 1 or 7 wherein the compound is contained in a composition which comprises a dried mixture of isolated anthocyanins, bioflavonoids and phenolics from *Prunus* and a food grade carrier.
11. The use according to claim 10 wherein the ratio of dried mixture to carrier is between about 0.1 to 100 and 100 to 0.1.



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12. The use according to claim 1 or 7 wherein the compound is incorporated into a food.

5 13. Use of an anthocyanin including cyanidin to inhibit inflammation in a mammal.

14. The use according to claim 13 wherein the anthocyanin is obtainable from *Prunus*.

10 15. The use according to claim 13 wherein the anthocyanin is obtainable from *Prunus avium*, *Prunus cerasus*, and mixtures thereof.

16. The use according to claim 13 wherein the mammal is human.

15 17. The use according to any one of claims 14, 15, or 16 wherein the anthocyanin is incorporated into a food.

18. The use according to any one of claims 14, 15, 16, or 17 wherein the anthocyanin is essentially pure.

20

19. Use of an bioflavonoid obtainable from *Prunus* to inhibit inflammation in a mammal.

20. Use of an phenolic obtainable from *Prunus* to inhibit inflammation in a mammal.

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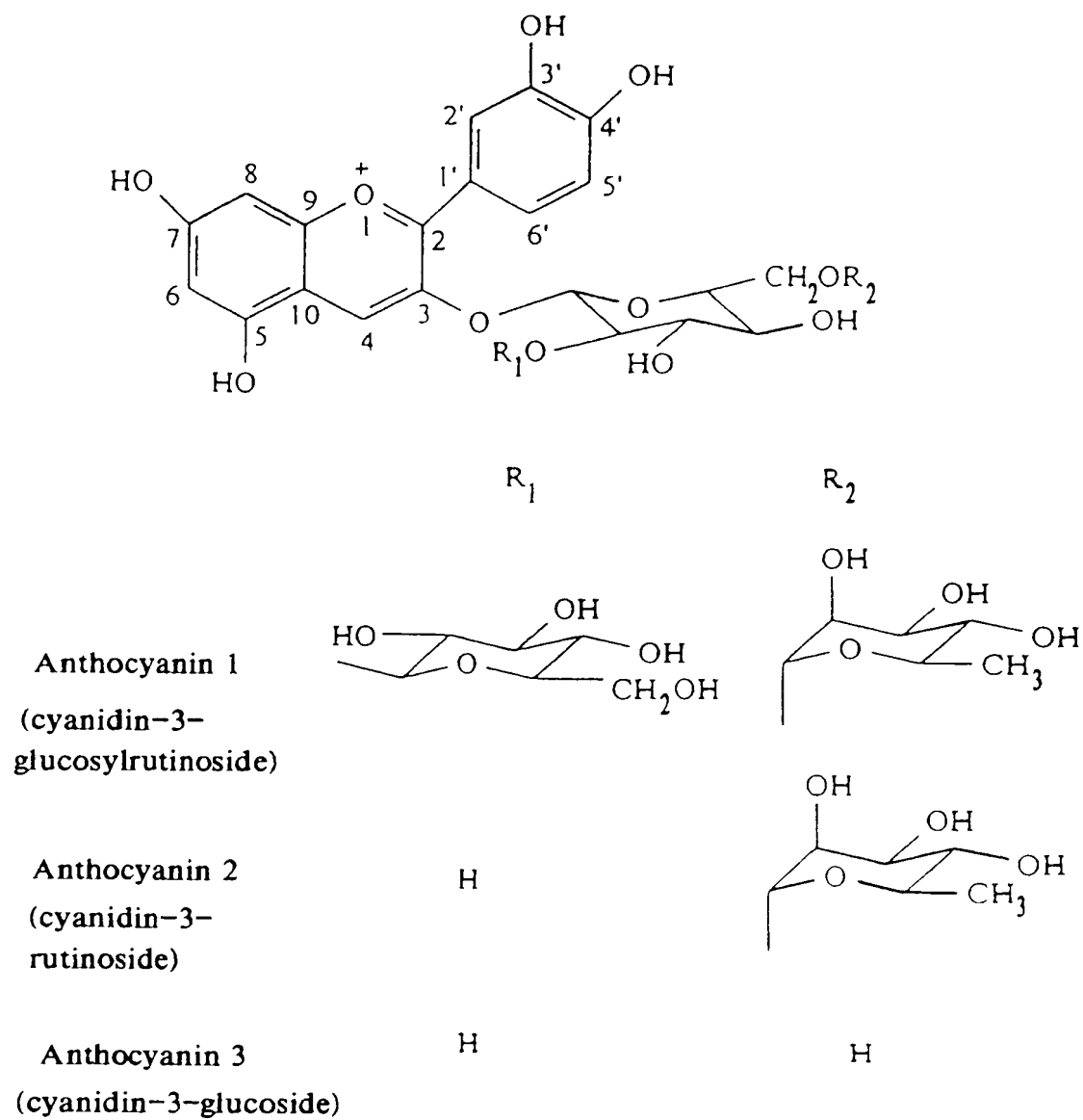
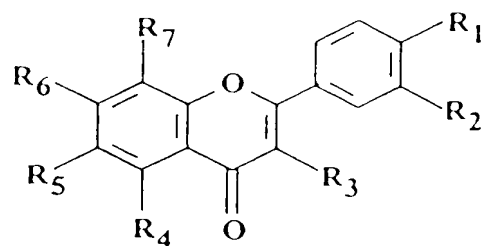


FIG. 1

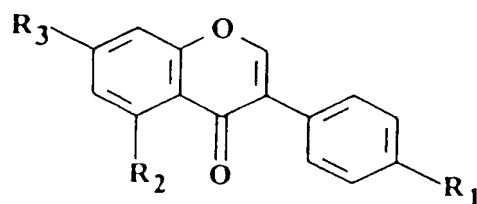


Compound	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>	R <sub>6</sub>	R <sub>7</sub>
Quercetin	OH	OH	OH	OH	H	OH	H
Kaempferol	OH	H	OH	OH	H	OH	H
Luteolin	OH	OH	H	OH	H	OH	H
Quercetrin 3-rhamnoside	OH	OH	rhamnose	OH	H	OH	H
Kaempferol 3-rutinoside	OH	H	rutinose	OH	H	OH	H
3'-methoxy kaempferol 3-rutinoside	OH	OMe	rutinose	OH	H	OH	H
5,8,4'-trihydroxyl-6,7-dimethoxyflavone	OH	H	H	OH	OMe	OMe	OH

FIGURE 2

SUBSTITUTE SHEET (RULE 26)

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Compound	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
Genistein	OH	OH	OH
Genistin	OH	OH	glucose
Biochanin A	OMe	OH	OH
Daidzein	OH	H	OH
Formononetin	OMe	H	OH

FIG. 3

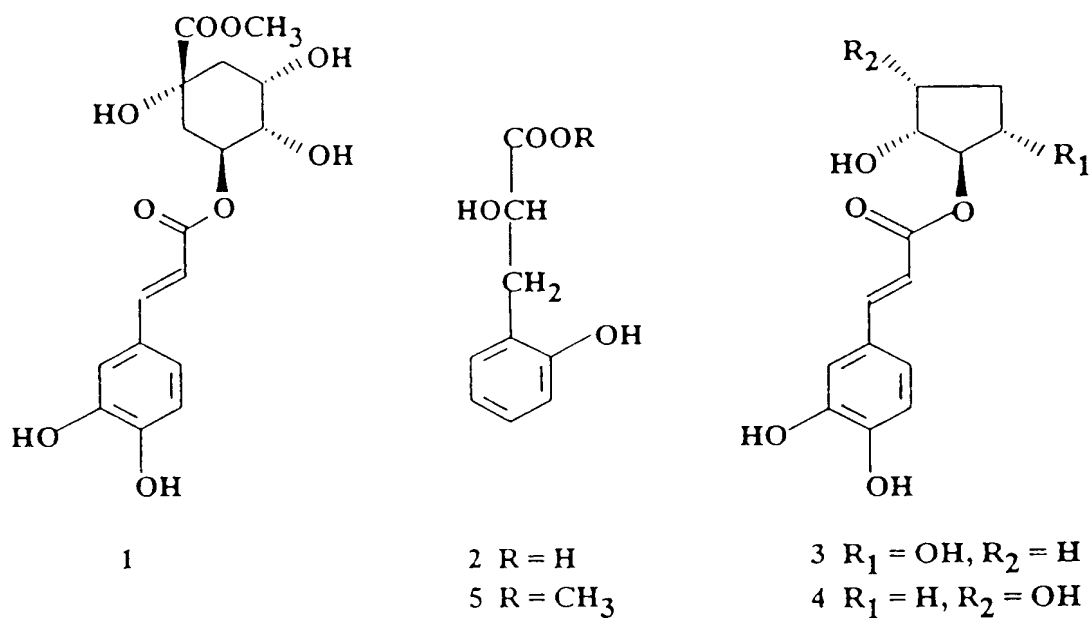


FIG. 4

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FIG. 5

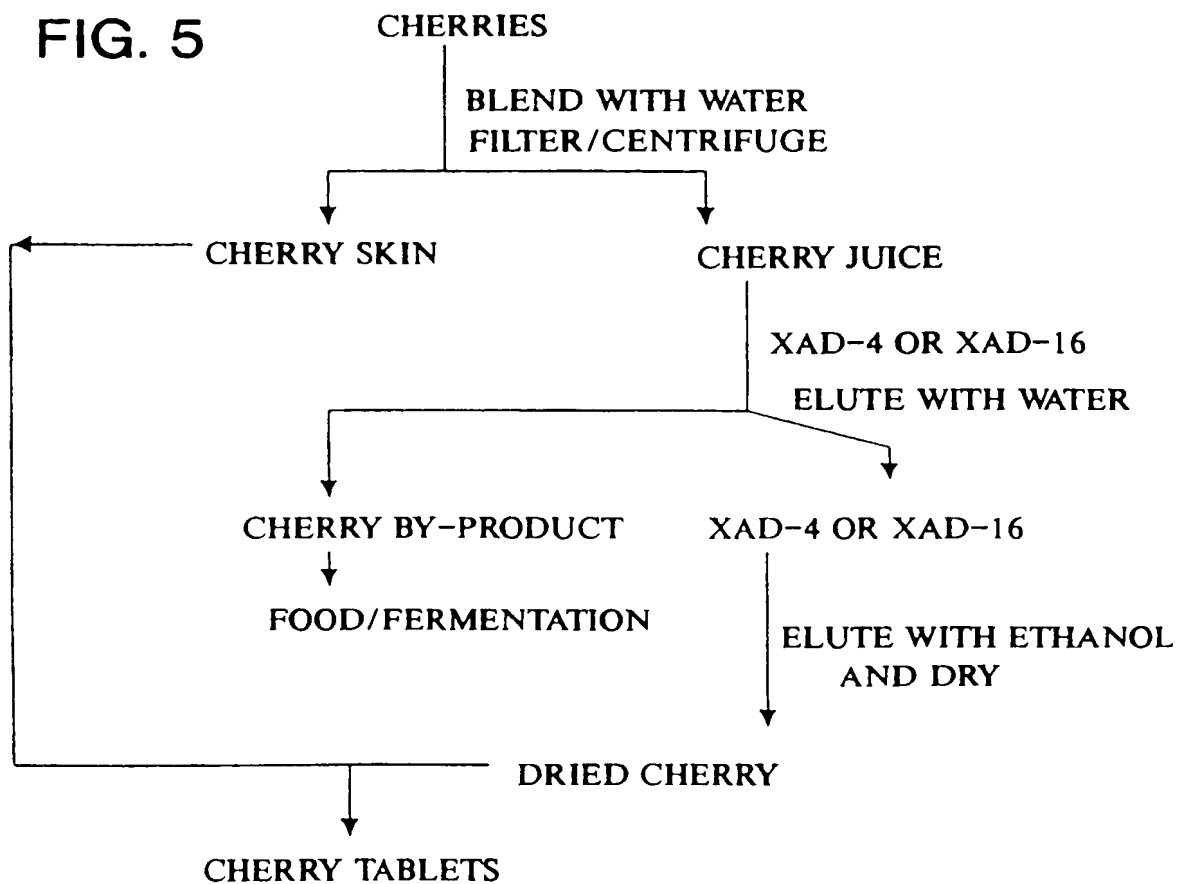
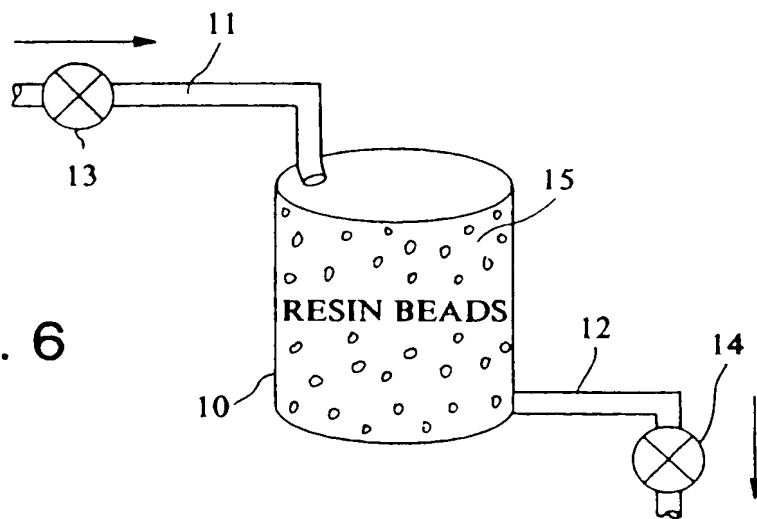


FIG. 6



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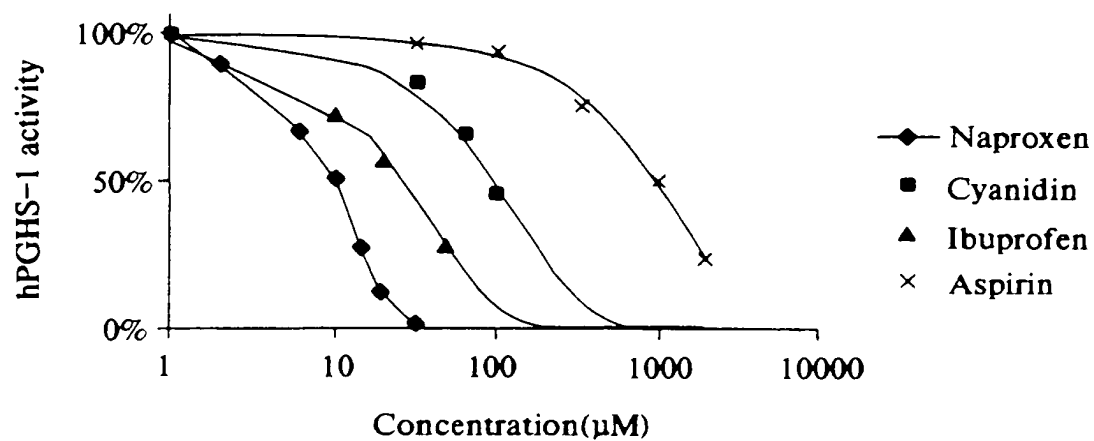


FIG. 7

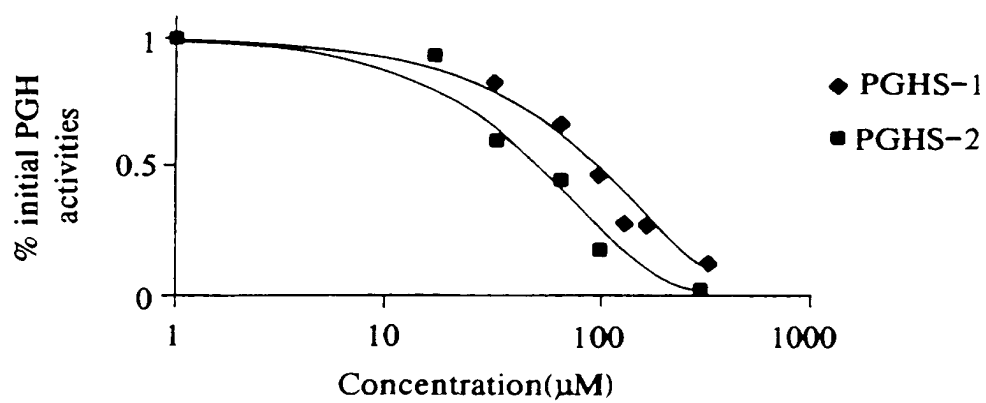


FIG. 8

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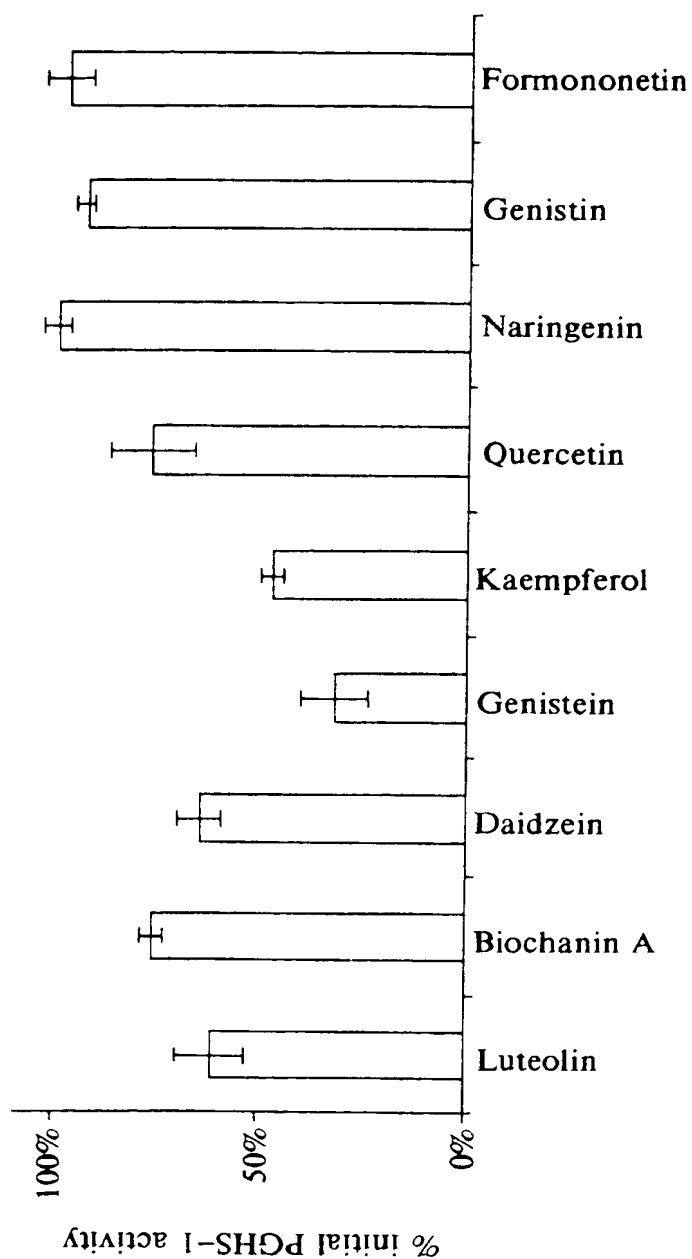


FIG. 9



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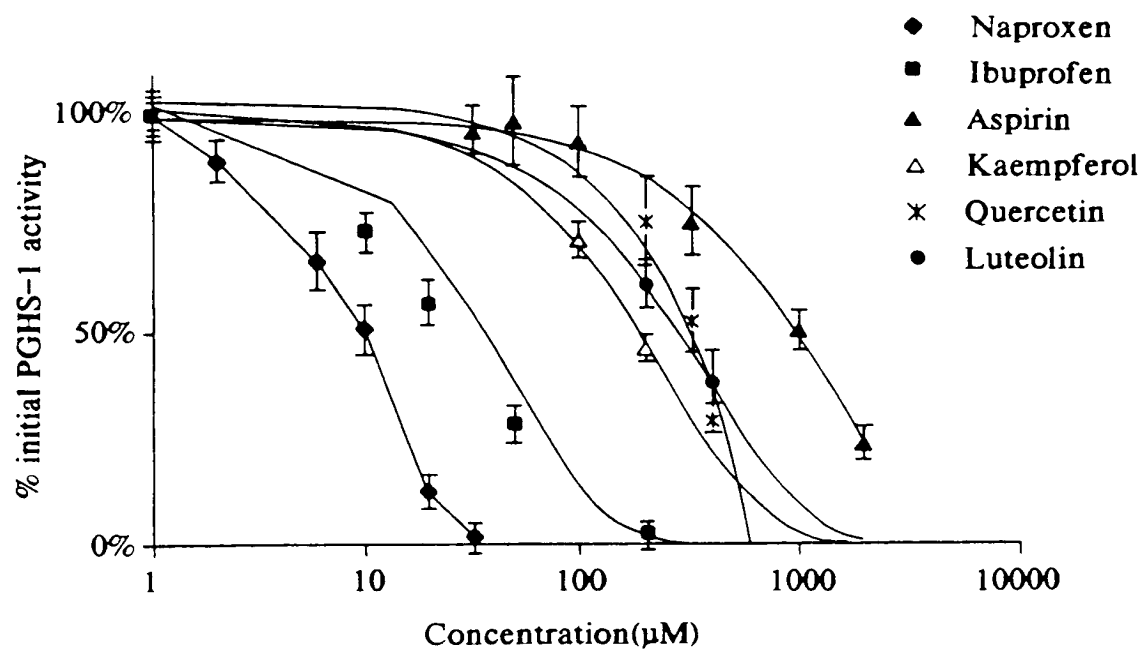


FIG. 10

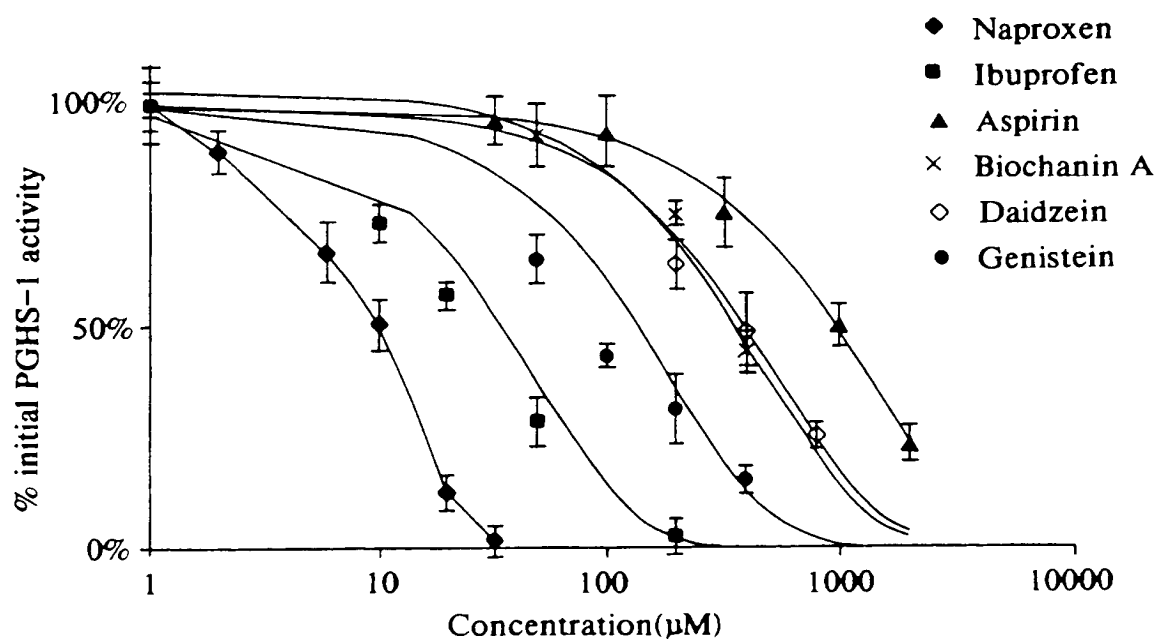


FIG. 11

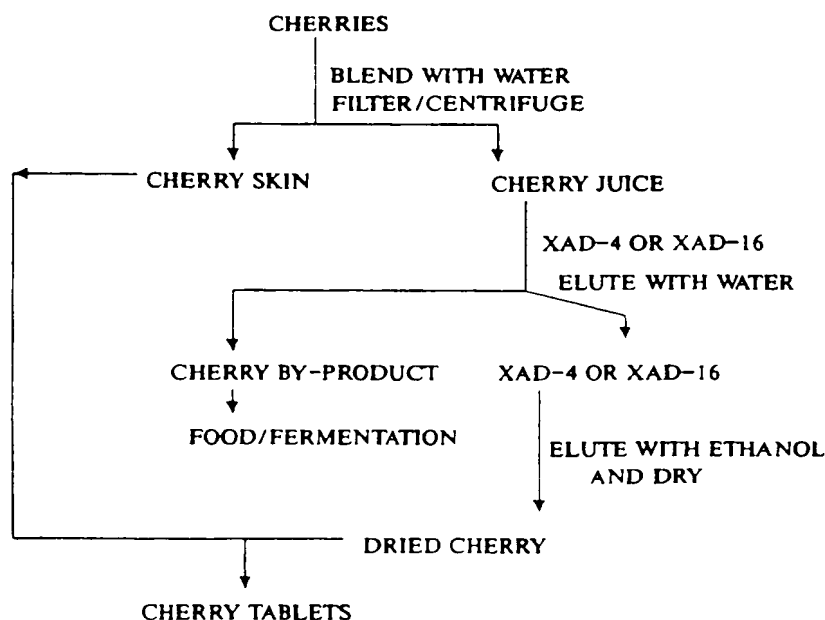




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(57) Abstract

A method for inhibiting cyclooxygenase enzymes and inflammation in a mammal using a cherry or cherry anthocyanins, bioflavonoids, and phenolics is described. In particular, a mixture including the anthocyanins, the bioflavonoids, and the phenolics is described for this use.

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## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K35/78 A61K31/352 A61K31/192 A61K31/075 A23L1/30  
A61P29/00 C07C69/74

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## B. FIELDS SEARCHED

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IPC 7 A61K A23L A61P C07C

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## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	GB 1 589 294 A (INVERNI DELLA BEFFA SPA) 13 May 1981 (1981-05-13) page 1, line 5 -page 1, line 30 page 2, line 20 -page 2, line 24 ----- -/--	1-20

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KIM KEE HEE ET AL: "Antiinflammatory activity of flavonoids: Mouse ear edema inhibition" ARCHIVES OF PHARMACAL RESEARCH (SEOUL), vol. 16, no. 1, 1993, pages 18-24, XP000886582 tables I-V concluding paragraph of the discussion ----	1-20
X	BLASZO G ET AL: "Anti-inflammatory effects of cherry (Prunus avium L.) stalk extract" PHARMAZIE, vol. 49, no. 7, 1994, pages 540-541, XP000887283 page 540 ----	1-20
X	DATABASE CAPLUS 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US SELENINA, L. V. ET AL: "Polyphenols of Potentilla erecta and their biological activity" retrieved from STN Database accession no. 80:22614 XP002137470 abstract & RAST. RESUR. (1973), 9(3), 409-14 , ----	1-20
X	DATABASE CAPLUS 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US RAZGA, ZSOLT ET AL: "Effect of benzopyranone derivatives on dithranol-induced ear edema in mice" retrieved from STN Database accession no. 110:127910 XP002137471 abstract & KISERL. ORVOSTUD. (1988), 40(6), 464-71, ----	1-20
X	LEE SONG JIN ET AL: "Antiinflammatory activity of isoflavonoids from Peuraria Radix and Biochanin A derivatives" ARCHIVES OF PHARMACAL RESEARCH (SEOUL), vol. 17, no. 1, 1994, pages 31-35, XP000886583 tables II,III ----- -/--	1-20

## INTERNATIONAL SEARCH REPORT

Inter. nat. Application No.

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HERNANDEZ-PEREZ M ET AL: "Phenolic composition of the mocan (Visnea mocanera)" J. AGRIC. FOOD CHEM., vol. 44, no. 11, 1996, pages 3512-3515, XP000632164 abstract -----	1-20
X	DE 197 20 767 A (PROTEKUM UMWELTINSTITUT GMBH O) 12 November 1998 (1998-11-12) column 1, line 3 -column 1, line 11 column 3, line 56 -column 4, line 30 examples 1-10 -----	1-20
A	CHANDRA A ET AL: "Isolation and stabilization of anthocyanins from tart cherries (Prunus cerasus L.)" J. AGRIC. FOOD CHEM., vol. 41, no. 7, 1993, pages 1062-1065, XP000381858 cited in the application the whole document -----	1-20

# INTERNATIONAL SEARCH REPORT

information on patent family members

Inter. nal Application No

PCT/US 99/29261

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 1589294 A	13-05-1981	BE 858522 A	02-01-1978
		DE 2740346 A	09-03-1978
		ES 462175 A	01-11-1978
		FR 2364030 A	07-04-1978
		GR 61176 A	03-10-1978
		JP 1648488 C	13-03-1992
		JP 3014286 B	26-02-1991
		JP 60011416 A	21-01-1985
		JP 1275525 C	31-07-1985
		JP 53062838 A	05-06-1978
		JP 59053883 B	27-12-1984
		PT 67009 A, B	01-10-1977
		US 4258055 A	24-03-1981
		US 4413004 A	01-11-1983
DE 19720767 A	12-11-1998	NONE	